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Detection of Respiratory Syncytial Virus RNA in Blood of Neonates by Polymerase Chain Reaction

Angela Rohwedder,[1]* Oliver Keminer,[1] Johannes Forster,[2] Katja Schneider,[2] Elisabeth Schneider,[2] and Hermann Werchau[1]

[1]Department of Medical Microbiology and Virology, Ruhr University, Bochum, Germany
[2]University Children's Hospital, Albert-Ludwigs University Freiburg, Germany

During the winter season of 1994/1995, nasopharyngeal aspirates and blood samples of neonates who were admitted to the Neonatal Intensive Care Unit (NICU) (group 1) and infants with respiratory tract disease (group 2) were examined prospectively for the presence of respiratory syncytial virus (RSV). Examination of nasal washes were done by antigen detection and blood samples were tested by nested reverse transcription and polymerase chain reaction (RT-PCR). The results of the 41 neonates studied were as follows: 14/41 were positive for RSV antigen in nasal washes and for RSV-RNA in blood, 5/41 were only RSV antigen positive. 13/41 neonates had negative nasal washes; 6 had positive RT-PCR results in blood. In 9/41 cases only blood samples were available. Five of these were positive by RT-PCR testing. Group 2 included 20 infants hospitalized with respiratory tract disease, e.g., pneumonia, bronchiolitis, or Upper Respiratory Tract Infection (URTI). Eleven out of twenty were positive for RSV antigen in nasal washes and 6/20 were also positive for RSV-RNA in blood. The conclusion is that viremia may be a frequent occurrence in neonates and young children. ***J. Med. Virol. 54:320–327, 1998.***

KEY WORDS: neonates; RSV infection; asymptomatic; viremia; nested RT/PCR

INTRODUCTION

Respiratory syncytial virus (RSV) is the most important viral respiratory pathogen in infants and young children. Most commonly, RSV causes upper respiratory tract disease. In 25% to 40% of such infections, however, the lower respiratory tract is involved, resulting in bronchiolitis or pneumonia. The most severe disease occurs during the first six months of life. However, there is a relative protection during the first five to six weeks, when maternally derived antibody titers are highest [Parrott et al., 1973; Holberg et al., 1991].

The mechanism by which RSV spreads from the upper to the lower respiratory tract is not clear. The route of cell-to-cell spread or of aspirated secretions may not completely explain the spread, because in experimentally infected animals the tracheal epithelium is only sparsely infected at any one time [Fishaut et al., 1978; Richardson et al., 1978]. Viremia has been implicated in the spread of other viral infections [Tyler and Fields, 1996]. However, thus far viremia has not been demonstrated during acute RSV infection of normal infants and children. Recently RSV antigens have been detected in circulating mononuclear leukocytes [Domurat et al., 1985]. Furthermore, RSV has been shown to replicate in mononuclear cells and macrophages in vitro [McCarthy et al., 1989; Midulla et al., 1989; Panuska et al., 1990; Cirino et al., 1993].

In order to assess the presence of viremia during the course of RSV infection, nasopharyngeal secretions and blood of 41 mostly preterm neonates (patient group 1) and 20 infants hospitalized with respiratory tract disease (patient group 2) were tested prospectively during the winter season of 1994/1995. Nasopharyngeal secretions were monitored by rapid RSV antigen detection using the Kallestad Pathfinder-Respiratory Syncytial Virus Direct Detection System and blood by a nested RT-PCR (Kallestad Pathfinder, RSV, Sanofi Diagnostics Pasteur, Chaska, MN).

MATERIALS AND METHODS

Study Design

Study patients included 33 preterm and 8 full-term infants in the NICU of the University Children's Hospital, Freiburg, and 20 infants hospitalized with suspected RSV lower respiratory tract infection between December 1994 and March 1995. Nasopharyngeal secretions for detection of RSV antigen were obtained

*Correspondence to: Angela Rohwedder, Department of Medical Microbiology and Virology, Ruhr University, Universitätsstrasse 150, 44801 Bochum, Germany. E-mail: Angela.Rohwedder@ruhr-uni-bochum.de

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weekly to separate virus positive and negative infants to prevent nosocomial infections.

At the same time, a weekly capillary blood sample was obtained from each child, if blood was drawn for other medical reasons. If the sample was taken in the range of two days before or after the day on which a nasopharyngeal specimen (NPS) was obtained, this was regarded as a set. Blood samples drawn on other days were not assigned to an NPS sample. A total of 92 NPS samples and 127 capillary blood samples were tested. The mean sample number per patient was equal in the group with positive and negative RSV test as well as in the neonatal group (patient group 1) and in the infants hospitalized with respiratory disease (patient group 2). This study was approved by the ethics committee.

The basic characteristics of the patients in group 1 and the presenting symptoms with RSV infection are shown in Table I. The clinical symptoms were recorded prospectively. Respiratory distress syndrome (RDS) and bronchopulmonary dysplasis (BPD) were classified on the basis of X-ray findings and cardiac defects on the basis of ultrasound examination. Of the clinical symptoms, only the most severe are listed in Table I, which included suspected sepsis, tracheitis (if tracheal suctions were purulent), and bronchitis (if the infant was ill enough to have an X-ray, on the exclusion of pneumonia). Bronchiolitis was based on the physical findings alone, as were signs of URTI. The latter mainly consisted of purulent nasal discharge or severe congestion of the nasal passages. Apnea and bradycardia were also observed in symptomatic infants.

Patients

Group 1 consisted of 41 neonates admitted to the neonatal intensive care unit; group 2 of 20 infants with respiratory tract disease.

Nasopharyngeal Secretions

Nasopharyngeal secretions were obtained by suction of both nostrils. Charrière 5 and 8 catheters were used in newborns and children, respectively. A mucus trap was attached. The catheter was inserted to a length equal to the distance between the nose tip and the entrance to the auditory channel. The catheter was slowly removed, sucking mucus with -2 N/m^2. The procedure was repeated in the other nostril. Most of the secretions stuck to the lumen of the catheter, and were transferred to the mucus trap by flushing with 1 ml PBS. The resulting material was stored for a maximum of 6 hours at 4°C until it could be tested for RSV antigen (Kallestad Pathfinder, RSV, Sanofi Diagnostics Pasteur). Positive samples were kept frozen at –70°C until further study.

Blood Samples

Blood samples were taken from blood gas determination samples. The blood was collected in 200-μl glass capillaries containing 50-IU natrium-heparinate (Clinitubes, Radiometer AS, Copenhagen, Denmark), which were centrifuged for 5 min with 2000 × g, to separate blood cells from plasma. After this procedure they were immediately frozen at –70°C. Blood cells and plasma were separately tested by RT-PCR.

RNA Isolation

Capillary blood and serum samples (approximately 50–100 μl) were recovered by pipetting with a capillarpipette tip, using the QIAGEN RNeasy kit to isolate total RNA from blood cells and the QuiAmp viral kit to isolate total RNA from serum. Total RNA was isolated from blood and plasma according to the instructions of the manufacturer (RNeasy Kit and QuiAmp viral kit, Qiagen, Germany).

Total RNA isolated from RSV-infected HEp-2 cells was used as control. HEp-2 cells were infected by RSV strain LONG. These controls were processed in the same way as the samples throughout RNA isolation, cDNA synthesis, and first- and second-step PCR. HEp-2 cells were cultured in DMEM medium (Gibco/BRL) supplemented with 10% fetal calf serum and an antibiotics mixture (250,000-U/l penicillin, 250-mg/l dihydrostreptomycin, 250-mg/l neomycinsulfate, 2,500-U/l bacitracin). The cultures were incubated at 37°C and then infected with RSV (moi = 0.1).

Reverse Transcription and Polymerase Chain Reaction

Reverse transcription and polymerase chain reaction (RT-PCR) were carried out as nested PCR. The cDNA reactions were undertaken in 25-μl reaction mixture containing 5-μl 5 × First-Strand Buffer (Gibco/BRL), 1-μl 0.1-M DTT (Gibco/BRL), 2-μl 5 mM of each dNTP (Pharmacia), 1-μl DMSO, 100-pmol primer, 5-μl isolated mRNA, and 5-U M-MLV-RT (Gibco/BRL). The reaction mixtures were incubated at 37°C for 40 min followed by 5 min at 95°C. The cDNA synthesis was primed separately for detection of RSV genomic RNA or mRNA of each gene.

First and second amplification reactions were carried out in a 100-μl reaction mix containing 50 pmol (20 pmol by nested PCRs) of each primer, 0.2 mM of each dNTP, 50-mM KCl, 20-mM Tris-HCl (pH 8.4), 1.5-mM $MgCl_2$, and 2-U Taq DNA polymerase (Gibco/BRL). In the first-step PCR, 10 μl of cDNA, and in the nested PCR, 2.5 μl of the first PCR were used as input. Samples were amplified in a thermal cycler (MJ Research) programmed for 40 cycles. The RSV-specific cDNA and PCR primers, cycle profiles, and the expected lengths are listed in Table II.

For all PCR amplifications, negative controls (water only) and positive controls (total RNA from RSV-infected HEp-2 cells; RSV strain LONG was used for infection of HEp-2 cells) were included. These controls were processed the same way as the samples throughout the cDNA synthesis and first- and second-step PCR. HEp-2 cells were cultured in DMEM medium (Gibco/BRL) supplemented with 10% fetal calf serum and an antibiotics mixture (250,000-U/l penicillin, 250-mg/l dihydrostreptomycin, 250-mg/l neomycinsulfate,

TABLE I. Clinical Characteristics of 31 Infected and 10 Uninfected Newborns and Results of Assays for RSV Infection (Patient Group 1)

Patient group 1	Test positive			Test negative
Age	< =30 days	>30 days	all	all
Number	22	9	31	10
Boys (n)	13	4	17	4
		Characteristics		
Gestational age (weeks) median (range)	30 (26–40)	32 (27–42)	31 (26–42)	30 (27–42)
		Manifestation		
Suspected sepsis	4	1	5	4
Pneumonia	0	0	0	0
Bronchiolitis	1	1	2	0
Tracheitis/bronchitis	5	3	8	3
URTI signs	4	4	8	3
Asymptomatic	8[a]	0	8	0[a]
		Tests		
Day of the relevant pair of tests: median (range) NPS	7 (2–30)	60 (31–323)	12 (2–323)	5 (2–79)
Positive/number tested	13/19 not tested: 3	6/7 not tested: 2	19/26 not tested: 5	0/6 not tested: 4
RT/PCR	19/22	7/9	26/31	0/10

[a]Patient groups compared regarding their similarity in terms of clinical characteristics: $P = 0.03$, Fisher's exact test, two sided.

TABLE II. Primer Sequences and Their Location

Primer	Sequence 5′–3′ direction	Position[a]	Cycle profile	Length, bp
NS 2-1 cDNA genomic RSV RNA	CCATGGACACAACCCACAATG	625–645	55°C/1′; 72°C/2′; 95°C/1′	379
NS 2-2 cDNA RSV mRNA	AATTTATGGATTGAGATCATAC	1004–983		
NS 2-3	GAGACCGTTGTCACTTGAGACC	680–701	60°C/1′; 72°C/2′; 95°C/1′	242
NS 2-4	GATTGATGAATATTGGCATAGGG	921–899		
N 1 cDNA genomic RSV RNA	TGCGATGTCTAGGTTAGGAAG	1345–1365	55°C/1′; 72°C/2′; 95°C/1′	411
N 2 cDNA RSV mRNA	GCTATGTCCTTGGGTAGTAAGCCT	1755–1732		
N 3	TGGAGTAGATGTAACAACACATCG	1417–1440	55°C/1′; 72°C/2′; 95°C/1′	212
N 4	CTATACATAATATTATCATCCCAC	1628–1605		
G 1 cDNA genomic RSV RNA	CCATTCTGGCAATGATAATCTC	4818–4839	55°C/1′; 72°C/2′; 95°C/1′	426
G 2 cDNA RSV mRNA	GTTTTTTGTTTGGTATTCTTTTGCAG	5242–5267		
G 3	CGGCAAACCACAAAGTCACAC	4878–4898	50°C/1′; 72°C/2′; 95°C/1′	326
G 4	GGGTACAAAGTTAAACACTTC	5183–5203		
F 1[b] cDNA genomic RSV RNA	GTTGGATCTGCAATCGCCAGTGGC	6090–6113	55°C/1′; 72°C/2′; 95°C/1′	539
F 2 cDNA RSV mRNA	GTACATAGAGGGGATGTGTG	6609–6628		
F 3	TTAACCAGCAAAGTGTTAGA	6222–6241	44°C/1′; 72°C/2′; 95°C/1′	242
F 4	TTTGTTATAGGCATATCATTG	6443–6463		

[a]According to the published sequence of RSV strain A2.
[b]Paton et al., 1992.

TABLE III. Results of Assays for RSV Infection of 20 Infants (Patient Group 2) Hospitalized With Pneumonia, Bronchiolitis, or URTI*

Admission diagnosis	Number	Mean age in days (range)	Mean days since onset of symptoms	NPS positive number (%)	PCR positive number (%)
Pneumonia	3	175 (117–205)	5	2 (67)	1 (33)
Bronchiolitis	11	131 (22–393)	5	6 (55)	3 (27)
URTI	6	113 (17–143)	4	3 (50)	2 (33)
Total	20	140 (17–393)		11 (55)	6 (30)

*RSV infection was detected by RSV antigen in nasopharyngeal secretions (NPS) by ELISA and RSV-RNA in blood by RT–PCR.

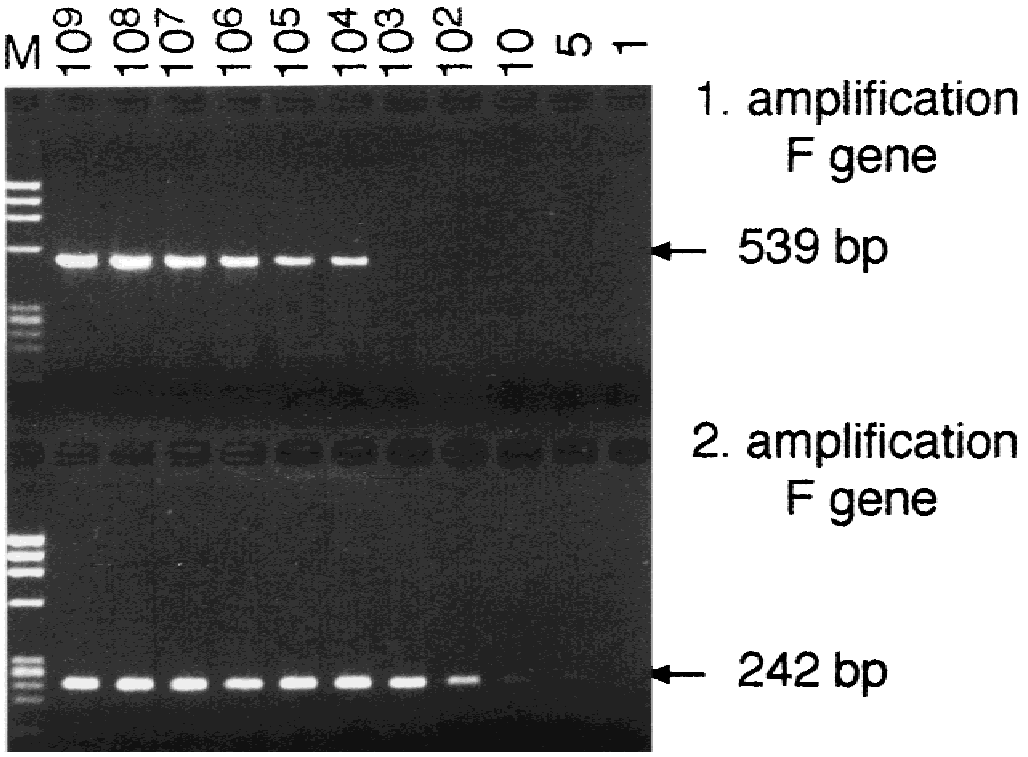


Fig. 1. Analytical sensitivity of reverse transcription (RT) and nested polymerase chain reaction (nPCR) for the detection of RSV-RNA of the F-gene. RNA of the synthetic transcripts was diluted to the number of copies indicated. The amplified PCR products of the first and second amplification were detected on a 2% agarose gel stained with ethidium bromide. Lane M = molecular weight marker PHI-X-RF-DNA digested with Hae III.

2,500-U/l bacitracin). The cultures were incubated at 37°C and then infected with RSV (moi = 0.1).

The PCR products (10 μl of each) were analyzed on 2% agarose gels stained with 1 μg/ml of ethidium bromide and the molecular size were compared with the molecular weight marker phi-X-RF-DNA digested with Hae III (Pharmacia, Freiburg, Germany).

The nested RT-PCR was established and optimized for each primer pair. To determine the sensitivity of the nested RT-PCR, definitive amounts of synthetic transcripts were used synthesized from a cloned cDNA of the corresponding gene (G-gene, F-gene, NS2-gene, N-gene). The RNA concentration was determined spectrophotometrically by UV A260 and then the number of transcripts was calculated. The detection limit of the qualitative nested RT-PCR was estimated to be one to five copies of the RSV target sequences.

Isolation and Cloning of the Amplification Products

Amplification products of the G-gene of some patients in both groups were recovered from agarose gels by using the gene clean II kit (Dianova, Germany) and then ligated to the pUC57/T cloning vector (Fermentas, Germany). This vector allows the direct cloning of PCR-products, as described by Marchuk et al. [1990]. After the transformation of competent bacteria (strain RRI), positive transformants were identified by restriction analysis of small-scale preparations of plasmid DNA [Sambrook et al., 1992].

Sequencing

Sequencing of cloned PCR-fragments was achieved by cycle sequencing with the Prism TM Ready Reaction Dye DeoxyTM Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and utilizing the universal and reverse M13 primer (Pharmacia) as sequencing primer. The sequencing reactions were analyzed on a 373 DNA sequencer (Applied Biosystems).

RESULTS

Clinical Characteristics of the Patient Groups

Forty-one neonates (patient group 1), of which 8 were full-term and 33 were prematurely born and twenty infants with respiratory tract disease (patient group 2), were studied. The mean gestational age of the neonates was 31 weeks, with a range of 26–42 weeks. Twenty-six (63%) of these became infected with RSV. The youngest baby to become infected was 2 days of age. Because the incubation period of RSV is 3 to 7 days, all infections except 2 were presumed to be nosocomially acquired. Fourteen out of 41 were positive for RSV antigen in nasal washes and demonstrated RSV-RNA in blood; 5 were only antigen positive. From 13 neonates with negative nasal washes, 6 had positive RT-PCR results

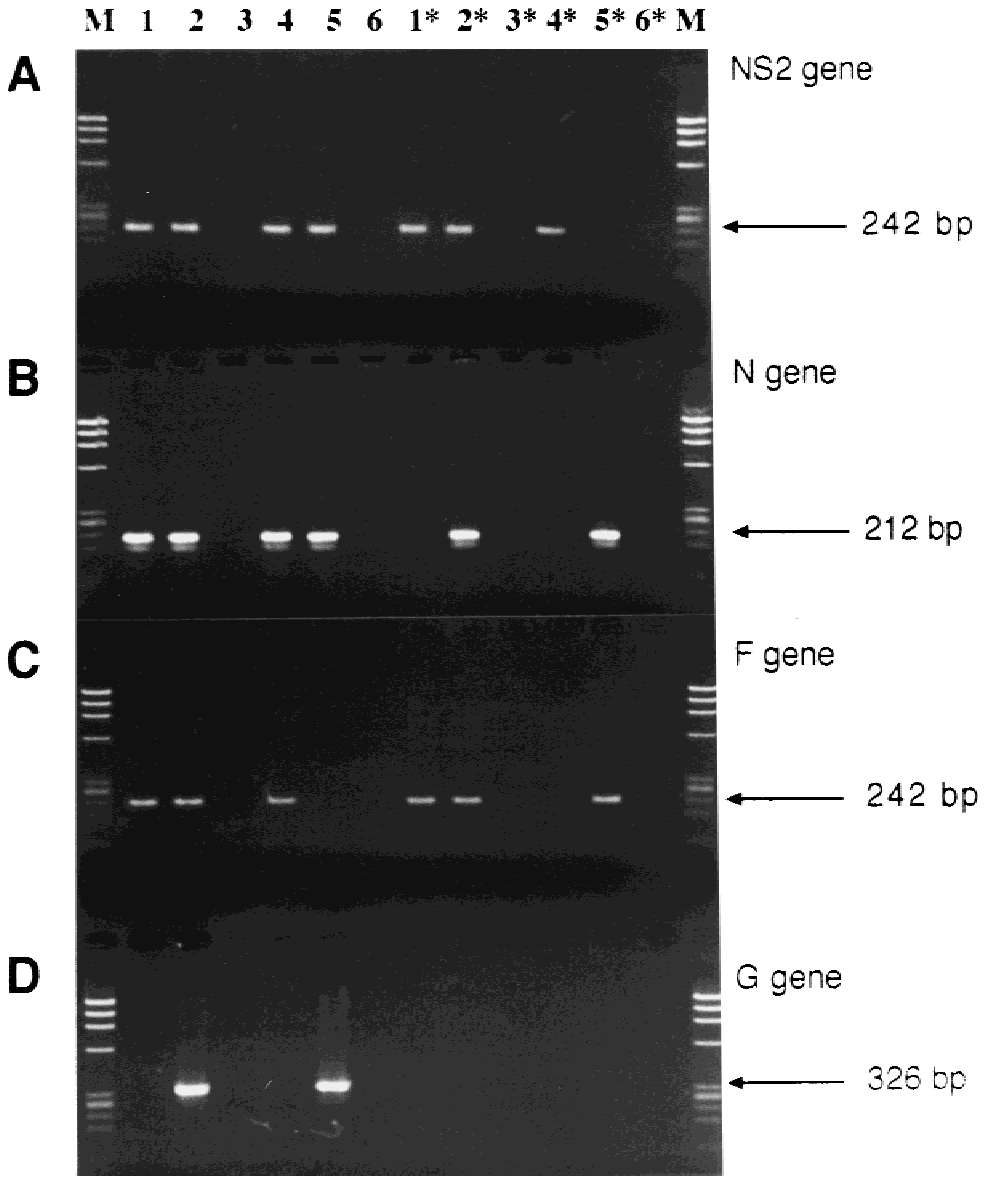


Fig. 2. Agarose gel electrophoresis of the nested polymerase chain reaction (PCR) products (from blood samples of six neonates, patient group 1). **A:** Amplified with primer pair NS2-1/NS2-2 (first amplification) and NS2-3/NS2-4 (nested amplification). Lane 1–6 shows detection of genomic RSV NS2-gene RNA; lane 1*–6*, detection of RSV NS2-gene mRNA; and lane M, molecular weight marker PHI-X-RF-DNA digested with Hae III. **B:** Amplified with primer pair N-1/N-2 (first amplification) and N-3/N-4 (nested amplification). Lane 1–6shows detection of genomic RSV N-gene RNA; lane 1*–6*, detection of RSV N-gene mRNA; and lane M, molecular weight marker PHI-X-RF-DNA digested with Hae III. **C:** Amplified with primer pair F-1/F-2 (first amplification) and F-3/F-4 (nested amplification). Lane 1–6 shows detection of genomic RSV F-gene RNA; lane 1*–6*, detection of RSV F-gene mRNA; and lane M, molecular weight marker PHI-X-RF-DNA digested with Hae III. **D:** Amplified with primer pair G-1/G-2 (first amplification) and G-3/G-4 (nested amplification). Lane 1–6 shows detection of genomic RSV G-gene RNA; lane 1*–6*, detection of RSV G-gene mRNA; and lane M, molecular weight marker PHI-X-RF-DNA digested with Hae III.

in blood. For 9 cases only blood samples were available. Five of these were positive by RT-PCR testing.

The clinical characteristics of the 31 babies who became infected as compared to the 10 who did not acquire RSV infection are shown in Table I. The frequency of underlying disease was not significantly different between infected and noninfected infants. The predominant clinical presentation or underlying diagnosis during hospitalization are also shown in Table I. Eight infected infants were considered to be totally asymptomatic.

In patient group 2, which included 20 infants with various respiratory tract presentations, e.g., pneumonia, bronchiolitis, or URTI, 11 were positive for RSV antigen in nasal washes and 6 were also positive for RSV-RNA in blood (Table III).

Detection of RSV-RNA

Nasopharyngeal secretions and blood samples of two patient groups were studied prospectively for the presence of RSV antigen or RSV-RNA. Whereas the naso-

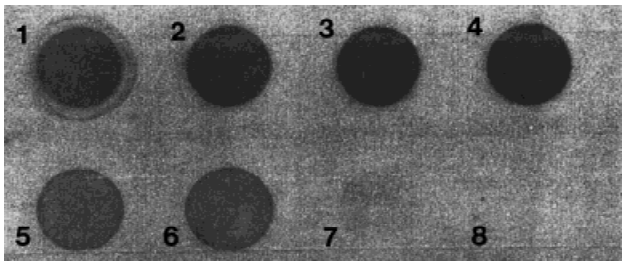


Fig. 3. Confirmation of the specificity of the PCR fragments. The specificity was determined by dot-blot hybridization. Some PCR products of the second amplification were dotted onto nylon membrane and then hybridized with a digoxigenin-labeled probe of the cloned N-gene (dots 2 to 6). PCR fragments of the controls were also dotted onto the nylon membrane (dot 1 = positive control; dots 7 and 8 = negative controls).

pharyngeal secretions were analyzed for RSV antigen utilizing a commercial RSV antigen detection kit, the blood samples were investigated using a highly sensitive nested RT-PCR assay. Using definitive amounts of synthetic transcripts synthesized from a cloned cDNA of the corresponding gene (G-gene, F-gene, NS2-gene, N-gene), the sensitivity of the nested RT-PCR was estimated to be one to five copies of the RSV target sequences. An example for estimation of the analytical sensitivity is given in Figure 1. This figure shows the result of one of four independent nested amplification experiments using ten- and twofold dilutions of synthetic RSV-RNA transcripts of the cloned F-gene.

Typical RT-PCR results of the analyzed samples are shown in Figure 2. For all samples tested, the first amplification reactions were negative (data not shown). Specific bands of the expected molecular weight were observed only with the nested PCR technique (Fig. 2A: NS2-gene; Fig. 2B: N-gene; Fig. 2C: F-gene; Fig. 2D: G-gene). As shown in Figure 2, not all samples were positive for all RSV genes investigated. There was a much higher frequency of detection for genes NS2, N, and F than for gene G. In only 3 out of 41 newborn blood samples was the G-gene identified. This low frequency of detection of product with G-gene primers could be related to sequence diversity.

To confirm the specificity of the PCR products, an aliquot of the first and second PCR assays were dotted onto nylon membrane and hybridized with a digoxigenin-labeled DNA fragment of the corresponding RSV gene. Figure 3 shows the dot-blot result of five samples that were positive for the N-gene after nested PCR (Fig. 2B).

Sequencing Analysis

In view of the potential for contamination by using nested PCR assays, the possibility of cross-contamination was examined with the reference strain RSV LONG by sequencing PCR products of the attachment protein G coding gene (G-gene). Analysis of the nucleotide and deduced amino acid sequences showed that all sequenced G-gene fragments were 100% identical. The sequence of the G-gene PCR product was designated "Freiburg." Figure 4 shows a multiple amino acid alignment of the detected sequence Freiburg with other RSV G-gene sequences. The comparison revealed that the sequence Freiburg had the closest homology to RSV isolate MAD-6-92 (95.8%), followed by the sequences of the isolates MON-8-92 (94.7%) and BIR-4-89 (94.7%). The lowest homologies were found in comparison with RSV strains A2 (80.9%) and LONG (83.3%).

DISCUSSION

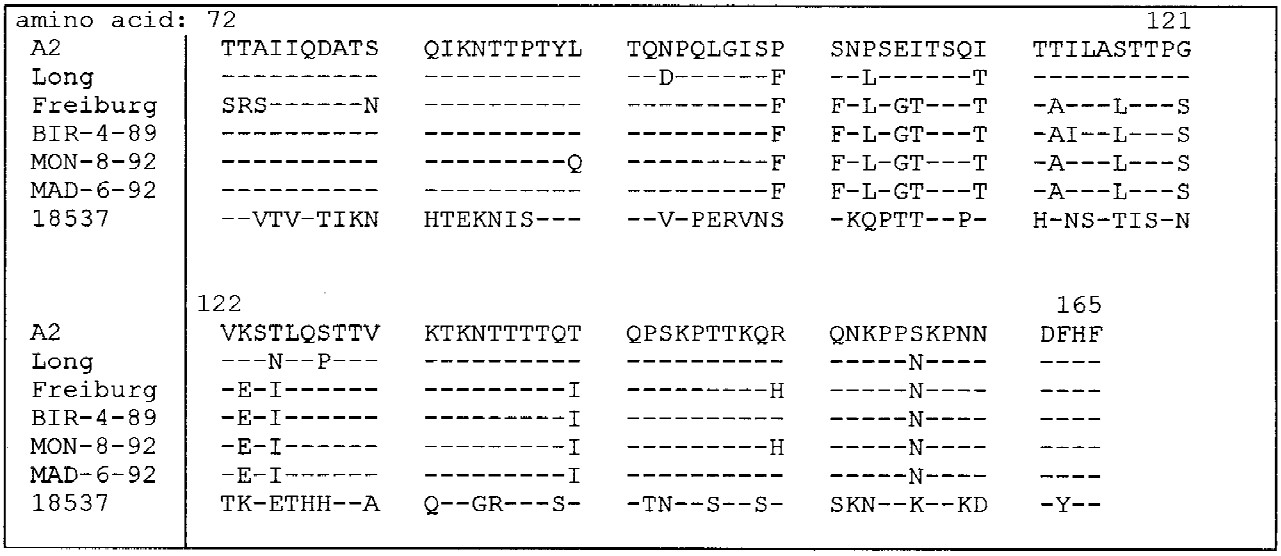
The data suggest that viremia may be present in neonates and young infants with RSV infection. Genomic and mRNA were detected in cells and plasma in 63% of neonates and 20% of young infants. We believe that the results are not due to PCR contamination, since precautions were taken to avoid this, and negative control reactions verified the absence of PCR contamination at all stages of the PCR process (Kwok and Higuchi, 1989). Carryover contamination between the patient samples did not occur because results with one primer set were retested and confirmed with primer sets for the detection of the RSV genes NS2, N, F, and G.

Since only the G-gene demonstrates the greatest antigenic and sequence differences between RSV strains [Cane and Pringle, 1995a, 1995b], the nucleotide and deduced amino acid sequences of the G-gene PCR products for all three samples were analyzed and aligned to known RSV. Comparison of the nucleotide and deduced amino acid sequences revealed unique sequences for all samples due to nosocomial infections. The unique sequence designated Freiburg was clearly distinct from that of the RSV strain LONG propagated in our laboratory.

The detection of genomes and transcripts in plasma is probably due to contamination by intracellular viral RNA from disrupted cells during the process of thawing the frozen samples. We therefore consider the viremia cell bound.

We believe that the RNA detected represents genomes and transcripts, and not merely defective RNA molecules removed by macrophages and in the process of elimination. Although tissue macrophages and dendritic cells at respiratory mucosal surfaces comprise a principal cellular element in the inactivation and clearance of most viral pathogens, they are either swallowed or they migrate to the regional lymph nodes where they present virus and viral antigens to T- and B-lymphocytes. It is unlikely that they gain direct access to the blood.

Because only frozen cells and plasma were available for testing, we were not able to identify precisely which cells actually contained viral RNA. We presume that candidates for RSV-bearing cells are monocytes/macrophages and lymphocytes, because these cells have been demonstrated to be infected by RSV in vivo [Domurat et al., 1985; Panuska et al., 1992] and in vitro [Midulla et al., 1989; McCarthy et al., 1989; Panuska et al., 1990; Cirino et al., 1993].



amino acid:	72				121
A2	TTAIIQDATS	QIKNTTPTYL	TQNPQLGISP	SNPSEITSQI	TTILASTTPG
Long	----------	----------	--D------F	--L------T	----------
Freiburg	SRS------N	----------	---------F	F-L-GT---T	-A---L---S
BIR-4-89	----------	----------	---------F	F-L-GT---T	-AI--L---S
MON-8-92	----------	---------Q	---------F	F-L-GT---T	-A---L---S
MAD-6-92	----------	----------	---------F	F-L-GT---T	-A---L---S
18537	--VTV-TIKN	HTEKNIS---	--V-PERVNS	-KQPTT--P-	H-NS-TIS-N

	122				165
A2	VKSTLQSTTV	KTKNTTTTQT	QPSKPTTKQR	QNKPPSKPNN	DFHF
Long	---N--P---	----------	----------	-----N----	----
Freiburg	-E-I------	---------I	---------H	-----N----	----
BIR-4-89	-E-I------	---------I	----------	-----N----	----
MON-8-92	-E-I------	---------I	---------H	-----N----	----
MAD-6-92	-E-I------	---------I	----------	-----N----	----
18537	TK-ETHH--A	Q--GR---S-	-TN--S--S-	SKN--K--KD	-Y--

Fig. 4. Comparison of the predicted amino acid sequence of the G-gene PCR product ("Freiburg") with other human respiratory syncytial virus G-gene amino acid sequences multiple-sequence alignment was generated with the computer program CLUSTAL W. Dashes denote identical amino acids. Sequences for the strains LONG, A2, and 18537 were taken from the date of Johnson et al. [1987] (accession numbers: Long = M17212, 18537 = M17213). Sequences for isolates BIR-4-89, Mon-8-92, and MAD-6-92 were taken from the EMBL database (accession numbers: BIR-4-89 = X73352, MON-8-92 = Z33430, MAD-6-92 = Z33418).

If mononuclear cells are infected in vivo they might contribute to pathogenesis of RSV disease by impairing both protective and curative immune responses [Collins et al., 1996; Kimpen and Heymans, 1993]. In infants with severely compromised immune function, for example, infected mononuclear cells could spread RS virus from the respiratory tract to other organs, such as kidney, liver, or myocardium [Fishaut et al., 1980; Milner et al., 1985].

The time of onset or the duration of the viremia was not defined because it was not possible to obtain daily matched nasopharyngeal and blood samples, nor posthospitalization samples. It is not known, therefore, whether the RSV sequences detected represent transient or persistent infections. To demonstrate persistence would require prospective study of individual patients showing that identical RSV sequences were obtained from serial samples over a period of time. This was not possible due to the difficulty in justifying repeated invasive sampling in these patients purely for research purposes. In assessing the duration of infection in BRSV-infected calves [Otto et al., 1996], it was observed that RSV-RNA was present in calf lung sections, in paratracheal lymph nodes, and in leukocytes six weeks following experimental infection [unpublished data].

RSV-RNA was more frequently detected in the cells of neonates than in older infants. This difference might be explained by age-related differences in susceptibility of cells such as monocytes/macrophages [Johnson, 1964; Hirsch et al., 197O]. Neonates may have higher amounts of passively transferred maternal RSV-specific antibodies than older children [Ogilve, 1981], which could facilitate the uptake of RSV antibody complexes via Fc-receptors of mononuclear cells [Krilov et al., 1989].

No correlation was found between viremia and the severity of the illness. Thus, the role of viremia in the pathogenesis of RSV disease in newborns and infants remains unknown.

From the clinical point of view, the most important finding was the significant number of infected but asymptomatic neonates revealed by this prospective assessment. There is only one other report in the literature [Hall et al., 1979] on such asymptomatic newborns. These children pose an epidemiological risk to other children in the ward. Likewise, preterm newborns with an atypical presentation must not be overlooked [Forster and Schuhmacher, 1995]. In a neonatal intensive care unit every effort must be taken to prevent the spread of infection, as these children are at special risk of complications.

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